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Sustained Exposure to the Widely Used Herbicide Atrazine: Altered Function and Loss of Neurons in Brain Monoamine Systems

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Abbreviations:

5-HIAA – 5 hydroxy indole acetic acid
5-HT – serotonin
ANOVA – analysis of variance
ATR – atrazine
COMT – catechol-O- methyltransferase
DA – dopamine
DBH – dopamine β-hydroxylase
DOPAC – dihydroxyphenylacetic acid
GABA – γ-aminobutyric acid
HPA – hypothalamo-pituitary-adenocortical axis
HPG – hypothalamo-pituitary-gonadal axis
HVA – homovanillic acid
LD_{50} – median lethal dose
LH – luteinizing hormone
LOAEL – lowest observed adverse effect level
MAO – monoamine oxidase
NE – norepinephrine
NOAEL – no observed adverse effect level
PRL – prolactin
RMANOVA – repeated measures analysis of variance
SNpc – substancia nigra pars compacta
T3 – triiodothyronine
TH^- – tyrosine hydroxylase negative cells
TH^+ – tyrosine hydroxylase positive cells
VTA –ventral tegmental area

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ABSTRACT

The widespread use of atrazine (ATR) and its persistence in the environment has resulted in documented human exposure. Alterations in hypothalamic catecholamines have been suggested as the mechanistic basis of the toxicity of ATR to hormonal systems in females and the reproductive tract in males. Since multiple catecholamine systems are present in brain, however, ATR could have far broader effects than currently understood. Catecholaminergic systems such as the two major long-length dopaminergic (DA) tracts of the central nervous system play key roles in mediating a wide array of critical behavioral functions. This study examined the hypothesis that ATR would adversely impact these brain DA systems. Male rats chronically exposed to 5 or 10 mg/kg ATR in diet for 6 months exhibited persistent hyperactivity and altered behavioral responsivity to amphetamine. Moreover, when measured two weeks post-termination of exposure, reductions in levels of various monoamines and loss of tyrosine hydroxylase positive (TH^+) and TH^- cells measured using unbiased stereology were found in both DA tracts. Acute exposures to 100 or 200 mg/kg ATR given i.p. to evaluate potential mechanisms were found to reduce both basal and potassium-evoked striatal DA release. Collectively, these studies demonstrate that ATR can produce neurotoxicity in DA systems that are critical to the mediation of movement as well as cognition and executive function. As such, ATR may be an environmental risk factor contributing to DA system disorders, underscoring the need for further investigation of its mechanism(s) of action and corresponding assessment of its associated human health risks.

INTRODUCTION

Atrazine (2-Chloro-4-ethylamino-6-isopropylamino-s-triazine), a chlorinated member of the family of s-substituted triazines, is one of the most widely employed herbicides in the world, with an estimated 76.4 million pounds used annually in the U.S. alone. It acts to suppress photosynthesis by inhibiting electron transfer at the reducing site of chloroplast complex II (Eldridge et al. 1999). Although it has limited solubility in water, ATR is frequently detected in ground and surface waters in agricultural regions (Colborn and Short 1999). Studies also reveal that ATR can be transported into the home, presumably tracked by soil (Lioy et al. 2000).

Human exposure has been confirmed (Adgate et al. 2001; Andrew Clayton et al. 2003), and, in fact approximately 60 % of United States population is exposed to ATR (Birnbaum and Fenton 2003). Recent reports indicate that acute dietary exposures range from 0.234 to 0.857 μg/kg/day, and corresponding figures for chronic dietary exposure are 0.046 to 0.286 μg/kg/day, considering all commodities with USEPA tolerances and drinking water (Gammon et al. 2005). Occupational exposure to ATR, as measured in mixer-loader-tender applicators, was reported to be approximately 2.8 mg ATR/day of work, with an absorbed dose of 1.8-6.1 μg/kg/day based on a 5.6% dermal absorption rate (Gammon et al. 2005). An earlier study of manufacturing workers reported a total ATR exposure of 10-700 μmol (~2.157-151.004 mg) per workshift (Catenacci et al. 1993).

The understanding of the potential of ATR to serve as a contributing factor to human disease and dysfunction is currently extremely limited. Epidemiologic studies have linked environmental and/or occupational ATR exposure to increased mortality

(Sathiakumar et al. 1996), and to Non-Hodgkin's lymphoma (MacLennan et al. 2003; Sathiakumar and Delzell 1997).

In experimental models, however, a growing experimental literature documents deleterious hormonal and reproductive system effects of ATR. In rodents, reported effects include reductions in testosterone levels, increases in triiodothyronine (T3), (Friedmann 2002; Stoker et al. 2002; Stoker et al. 2000), suppression of immune function (Rooney et al. 2003), of luteinizing hormone and of prolactin surges (Cooper et al. 2000), the appearance of mammary gland tumors, a disruption of regular ovarian cycles, and the induction of pseudopregnancies (Cooper et al. 1996; Laws et al. 2000).

The effects of ATR on ovarian function in female rats have been ascribed to changes in function of catecholamines in the hypothalamus, specifically decreases in norepinephrine (NE) and increases in dopamine (DA) in this region (Cooper et al. 1998). In correspondence with this observation, *in vitro* studies in PC12 cells show concentration-dependent decreases in intracellular DA after exposure to 12.5-200 uM ATR for 6, 12, 18 and 24 hours and decreases in NE release and intracellular NE concentrations after exposures to 100 and 200 uM for 12, 18 and 24 hours (Das et al. 2000; Das et al. 2003). In addition, reductions in the expression of DA ß-hydroxylase (but not of tyrosine hydroxylase) were observed. The inhibitory effects of ATR on intracellular NE content and NE release, but not on DA intracellular content, were reversed when PC12 cells were coincubated with ATR and agents known to enhance transcription, phosphorylation, or activity of tyrosine hydroxylase and DA ß-hydroxylase, such as 8-bromo cAMP, forskolin or dexamethasone (Das et al. 2003). These findings suggest that ATR could disrupt catecholamine metabolism by altering its biosynthetic

enzymes.

The fact that ATR can adversely impact hypothalamic catecholamine systems has notable implications, since such effects would be unlikely to be restricted to this particular region, but could affect brain catecholamine systems more generally, and thus impact pathways critical to the control of movement (nigrostriatal DA systems) and of complex cognitive functions (mesocorticolimbic DA systems). If so, then ATR exposures may also serve as a risk factor for neurodegenerative diseases and/or dysfunctions associated with these systems, which include Parkinson's disease, schizophrenia, and attention deficit disorder among others (Crossman 2000; Epstein et al. 1999; Viggiano et al. 2003). Indeed, epidemiological studies have linked pesticides to an increased odds ratio for Parkinson's disease (Breysse et al. 2002), and various pesticides that impact catecholaminergic systems have been shown to produce characteristics of Parkinson's disease in experimental models (Betarbet et al. 2000; Reeves et al. 2003; Thiruchelvam et al. 2000b).

The potential for neurotoxic effects of ATR *in vivo* however, particularly chronic effects, has received almost no experimental attention. Oral exposure of rats to 1000 mg/kg ATR for 4-11 days decreased rearing in the open field (Ugazio et al. 1991) while acute exposure of rats to 100 mg/kg decreased spontaneous purkinje cell firing rate and cerebellar potential evoked by electrical stimulation (Podda et al. 1997).

The objective of the current study was to evaluate the potential for sustained low-level ATR exposure to impact two critical catecholamine pathways of the brain, specifically, the nigrostriatal DAergic pathway, involved in the mediation of movement (Crossman 2000), and the mesocorticolimbic DA pathway critical to complex cognitive

functions (Clark et al. 2004; Remy and Samson 2003). For this purpose, locomotor activity was evaluated across the course of exposure, while monoamine levels in striatum, prefrontal cortex, nucleus accumbens and hypothalamus, and stereological cell counts of tyrosine hydroxylase positive (TH^+) and negative (TH^-) cells in the midbrain were evaluated 2 weeks after cessation of exposure. Further, this study sought to determine mechanisms by which any changes in DAergic function in these pathways might be produced by examining the acute effects of ATR on striatal DA release using microdialysis.

MATERIALS AND METHODS

Chronic Atrazine Exposure

Subjects, Exposure and Experimental Design

Thirty male Long-Evans rats purchased from Taconic Farms (Germantown, NY) were housed individually in plastic cages in a temperature and humidity-controlled vivarium room with a 12 hour dark-light cycle (6 A.M lights on). Food intake was restricted to maintain body weights at 300 g and water was available ad libitum during the entire experiment. In our experience, this protocol sustains health and viability to a greater degree than does ad libitum feeding. At nine months of age, exposure to either 0, 5 or 10 mg/kg ATR mixed in food was initiated with continuation of ad libitum access to distilled drinking water. These doses of ATR were chosen based on reports for the rat of an oral LD_{50} value of 1869 mg/kg (U.S. EPA 2001), a NOAEL of 3.3 mg/kg/day and a LOAEL of 34.5 mg/kg /day for this route of administration measured as body weight loss. A chronic dietary NOAEL of 1.8 mg/kg/day and LOAEL of 3.65 mg/kg/day were also reported (U.S. EPA 2001). Body weights and food consumption were recorded periodically over the entire duration of the experiment. All procedures were carried out in accord with National Institutes of Health and University of Medicine and Dentistry of New Jersey Animal Use and Care Committee Guidelines. The experimental design is summarized in Figure 1A.

Locomotor activity was recorded at two, three, and six months of ATR exposure and 2 weeks following cessation of exposure. At the two month time point, locomotor activity was measured on three consecutive days with animals receiving an i.p. injection of saline five minutes before the session during the first two days, and an injection of d-

amphetamine sulfate (1 mg/kg) on day three. Only a single locomotor activity session was carried out at the 3 and 6 month time points and at two weeks following the termination of ATR exposure. Locomotor activity was recorded during the light phase (from 9 A.M to 1 P.M) of the light-dark cycle using methods described below.

Two weeks after cessation of ATR exposure, rats were sacrificed by decapitation, brains removed and hypothalamus, prefrontal cortex, nucleus accumbens and striatum were dissected on ice and frozen for HPLC analysis. The remaining tissue was postfixed in 4 % paraformaldehyde for immunohistochemistry and stereological counts.

Locomotor Activity Measurement

Each rat was individually placed in an automated locomotor activity chamber equipped with infrared photobeams (Opto-Varimex Minor; Columbus Instruments International Corporation, Columbus, OH). Horizontal, vertical and ambulatory activities were simultaneously measured and data collected over the course of a 45-minute session.

Measurement of Monoamine Levels

Tissues were sonicated in 0.1N perchloric acid and centrifuged. Supernatants were stored at –80 ° C until analyzed for monoamine content. Pellets were digested in 0.5 M NaOH for measurements of protein concentration using Bio-Rad reagents (Hercules, CA).

Monoamines and their metabolites were measured by HPLC with electrochemical detection as described elsewhere (Thiruchelvam et al. 2000a). Briefly, a Waters pump 515 plus autosampler (Waters Corporation, Milford, MA) was joined to a chromatographic column (Alltech Associates Inc, Deerfield, IL). The amperometric potential was set at 600 mV relative to the Ag/AgCl, and the sensitivity of the detector

was set at 100 ρA (microdialysates) or 1 ηA (tissue samples). The mobile phase was an isocratic 0.1 M monobasic phosphate solution containing 0.5 mM sodium octyl sulphate, 0.03 mM EDTA and 12-14% v/v of methanol. Results generated by these determinations were analyzed with Empower Pro program (Empower Software, 2002, Waters Corporation) and are expressed in ρg/ml of microdialysate or ηg/mg of protein of tissue. DA turnover was expressed as the ratio DOPAC/DA.

Tyrosine Hydroxylase Immunohistochemistry

Five randomly selected paraformaldehyde fixed brains from each treatment group were cut into 30 μm sections and collected in cryoprotectant and stored at –20 ° C for immunolabeling studies. Sections were rinsed with 0.1 M PB, blocked with 10% normal goat serum for non-specific binding and incubated in TH primary antibody (Chemicon, Tamecula, CA) for 48 hours at a dilution of 1:3500 in PB with 0.3 % Triton X-100 and 10% normal goat serum. Consequently sections were incubated with a secondary antibody 1:200 (Vector Laboratories, Inc, Burlingame, CA) overnight. Sections were washed and incubated with avidin-biotin solution from Vectastain ABC reagents (Vector Laboratories) for 1 hour and developed in 3-3'-diamonobenzidine tetrachloride and H_2O_2 in 0.05 M Tris buffer. Sections were counterstained with cresyl violet following TH staining. Total numbers of TH^+ and Nissl stained neurons (TH^-) in substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) were counted using the optical fractionator method as described below.

Stereological Analysis

After delineation of the SNpc and VTA at low magnification (4X objective), one side of every fourth section from the entire midbrain region was sampled at higher

magnification (100X objective) using the stereology module of the Stereo Investigator imaging program (MicroBrightField, Inc., Williston, VT) with an Olympus Provis microscope. The optical fractionator method, an unbiased quantitative technique, was used for counting TH^+ (TH-positive and cresyl violet positive neurons) and TH^- (cresyl violet positive only) cells. Criteria for TH^+ and TH^- neurons were determined as previously described (Barlow et al. 2004; Thiruchelvam et al. 2004). The mean thickness was determined by measuring two fields from five sections per sample, and the entire depth of field was sampled, ignoring the upper and lower 0.5 μm. All samples were evaluated by one experimenter without knowledge of treatment status.

Chemicals

Atrazine (2-Chloro-4-ethylamino-6-isopropylamino-s-triazine) at 98% purity was purchased from Chem Services, Inc (West Chester, PA). Reagents for microdialysis, HPLC analysis, methylcellulose, and cresyl violet were purchased from Sigma (St Louis, MO).

Acute Atrazine Exposure

Subjects, Exposure and Experimental Design

Thirty male Long Evans rats weighing between 270-320 g purchased from Taconic Farms (Germantown, NY) were habituated to constant standard laboratory conditions of humidity, temperature and dark-light cycle (6 A.M lights on) as described above. As shown in Figure 1B, microdialysis was used to evaluate changes in striatal DA release following acute i.p. exposures to ATR in sessions lasting 7 hours.

Surgery

After a habituation period of at least one week, rats were anesthetized with

pentobarbital (30-40 mg/kg i.p.) and every half hour thereafter received an injection of atropine sulfate (0.3 mg/kg i.p.) to avoid respiratory failure during the cannula implantation. Once anesthetized (assessed by absence of corneal reflex), the rat was placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), the skull exposed, and a hole was drilled for placement of a guide cannula (MD-2250, Bioanalytical Systems, Inc, West Lafayette, IN) over the right striatum, using stereotaxic coordinates (AP: +1.0 mm; ML: -2.0 mm with reference to bregma, and DV: -3.4 mm from flat skull), according to the atlas of Paxinos and Watson (Paxinos and Watson 1986). The cannula was fixed to the skull with anchor screws and acrylic cement. After surgery, rats were individually housed for a recovery period of 5- 7 days with food restricted to keep body weight at 300 g and water was available ad libitum.

Microdialysis

A probe of concentric design (MD-2262 tip 2 mm, Bioanalytical Systems, Inc) was inserted into the guide cannula. The dialysis probe was continuously perfused at a flow rate of 2.5 μl/min through a liquid swivel from an automated system (Bioanalytical Systems, Inc) with a physiological Ringer's solution containing 147 mM NaCl, 4.0 mM KCl, 1.2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.0-6.5. Sample collection occurred every 30 minutes. The first hour of sampling was discarded to avoid erroneous data due to probe insertion. After three baseline samples, rats received an i.p. injection of vehicle (1% methylcellulose) or ATR (100 or 200 mg/kg), and five subsequent samples of perfusate were collected. In order to probe characteristics of DA release, a high potassium solution (91 mM NaCl, 60 mM KCl, and 1.2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.0-6.5) replaced the normal Ringer's solution and two samples were collected under these conditions.

Normal Ringer's solution was subsequently restored and two additional samples of perfusate collected. Collection vials contained 3.75 µl of 0.1 M $HClO_4$ solution. Collected samples were immediately frozen at –80 °C until monoamine quantification by HPLC as described above.

Histology

At the completion of microdialysis sampling, rats were overdosed with sodium pentobarbital and transcardiacally perfused with an isotonic saline solution followed by 10 % formaldehyde. Brains were postfixed in 10 % formalin overnight, and subsequently transferred to 30 % sucrose. Brains were sectioned in 50 µm coronal slices, mounted, stained with cresyl violet and cover-slipped. Cannula placement for the microdialysis study was confirmed under microscopic analyses.

Statistical Analyses

Total locomotor activity counts, body weight and food consumption were analyzed using repeated measures analysis of variance (RMANOVA; treatment by time) followed by post hoc tests as appropriate. Responsivity to d-amphetamine and changes in neurotransmitter levels in various brain regions were evaluated by one-way ANOVA with post hoc assessments in the event of main effects of treatment. To provide a more conservative analysis of changes in cell counts, since counts in both regions were derived from the same animals (brains), RMANOVA was carried out based on changes in TH^+ and TH^- cells in both substantia nigra and ventral tegmental area (but not total counts since that was the sum of the TH^+ and TH^- cells), followed by post-hoc testing as appropriate. Effects of ATR on microdialysis were evaluated by RMANOVAs with treatment and time as factors, followed by post-hoc evaluation in the case of main effects

or interactions. In all cases, statistical significance was defined as $p \leq 0.05$.

RESULTS

Chronic Atrazine Exposure

Gross Effects of Treatment

No treatment-related changes in body weight or food consumption were detected at any point during the course of the exposure (data not shown), nor were any other signs of overt toxicity manifest at any point.

Locomotor Activity

In contrast to the other time points of measurement, the assessment of locomotor activity after 2 months of ATR exposure actually involved three sessions, the first two of which were preceded by an i.p. injection of saline and the third by 1 mg/kg d-amphetamine sulfate. No treatment-related changes in locomotor activity were seen in either of the sessions preceded by saline. However, in the third session, the administration of d-amphetamine increased locomotor activity of all three groups relative to levels of activity following saline administration (session two) [$F(2,26)= 3.63$, $p <0.041$]. Additionally, these increases were modified by ATR treatment in that the 10 mg/kg dose further enhanced locomotor activity by an additional 70% (Figure 2A) relative to the increases in the 0 and 5 mg/kg groups, as confirmed in post hoc analyses.

At the remaining time points of measurement, single locomotor activity sessions were carried out in the absence of drug administration. Under these conditions, after three months of ATR exposure, pronounced increases in locomotor activity were found again at the 10 mg/kg dose of ATR [$F(2,26)= 3.62$, $p=0.041$], with levels of horizontal activity that exceeded those of controls and the 5 mg/kg group by approximately 50% (Figure 2B). These treatment-related effects were again evident in the measurement of locomotor

activity at the six month time point [F (2, 24) = 3.45, p=0.048] and again when measured two weeks following the termination of ATR treatment [F (2, 24) = 4.42, p=0.024] where levels remained at 40% above control.

Changes in Monoamine Levels

Measured two weeks following the termination of ATR exposure, significant changes in DA content (Figure 3A) were detected in striatum [F (2,23)= 3.61, p=0.044] as well as in frontal cortex [F (2,21)=3.82, p=0.039]. Statistical analysis confirmed decreased levels of DA (approximately 20%) in relation to treatment in striatum, with post-hoc assessments indicating efficacy at the 10 mg/kg ATR dose with a similar although non-significant trend at 5 mg/kg. In contrast, levels of DA were increased in prefrontal cortex in an inverse U-shaped fashion, with post-hoc assessment confirming a significant increase at 5 mg/kg (by 30-40%) with levels declining back toward control values at 10 mg/kg. Both doses of ATR reduced levels serotonin in hypothalamus (Figure 3B) [F (2, 21) = 5.19, p=0.015]) by levels of 10-15%. Chronic ATR exposure also decreased levels of NE in frontal cortex (Figure 3C) [F (2, 21) = 3.84, p=0.038], with post-hoc assessments showing this effect occurring at the 10 mg/kg dose producing reductions of approximately 15-20%. While a trend towards increases in NE in nucleus accumbens was suggested, it was associated with significant variability and therefore not statistically significant.

No changes in levels of the metabolites of either serotonin (5-HIAA) or DA (DOPAC, HVA) or DA turnover (DOPAC/DA) were observed in any region.

Unbiased Stereological Counts of Cells in the Midbrain

Changes in numbers of cells in the regions of the cell bodies of the two major DA pathways are shown in Figure 4 for a sample of 5 randomly selected animals from each treatment group. The top panel shows numbers of TH^+, TH^- and total cells in substantia nigra pars compacta, while corresponding data for the ventral tegmental area are presented in the bottom row. Given that these regions were obtained from the same brains, a more conservative statistical analysis based on RMANOVA was carried out to examine the impact of treatment on numbers of cells utilizing counts of TH^+ and TH^- from each region (not including total counts). That analyses confirmed a significant main effect of treatment ($F(2, 36) = 5.53$, $p=0.02$), and no interaction of treatment by region, indicating that cell loss occurred in both regions, and moreover, in both TH^+ and TH^- cells. These effects were primarily attributable to the 10 mg/kg dose of ATR, as confirmed in subsequent post-hoc tests, where the mean reductions in cell numbers ranged from 9-13%, while those in the 5 mg/kg group ranged from 0-3%.

Acute Atrazine Exposure

That systemic administration of ATR can indeed directly affect brain DA systems was further confirmed in microdialysis experiments. The impact of acute i.p. administration of ATR (100 or 200 mg/kg) on levels of DA in striatum as assessed via microdialysis are presented in Figure 5A. Acutely, ATR significantly decreased basal DA release, as shown in the inset in panel A (main effect of treatment [$F(2, 19) = 4.88$, $p=0.02$], sampling time [$F(9, 18) = 26.77$, $p < 0.0001$] and treatment by time interaction [$F(18,171) = 2.77$, $p=0.0003$]). Post-hoc tests confirmed decreases as measured at 90,

120, 150 and 180 minutes post administration of ATR. By 150 minutes, the decrements averaged approximately 40% and were seen in both the 100 mg/kg and the 200 mg/kg treated groups.

A dose-dependent decrease in DA release was also observed when the system was challenged with 60 mM high potassium solution for 60 minutes [$F(2, 19) = 3.717$, $p=0.0434$]. While the control group showed a 1256% increase from baseline in response to potassium (time point 210 min), corresponding values for the 100 mg /kg and 200 mg /kg ATR groups were 729 % and 427%, respectively, from baseline. After high potassium perfusion, the system was flushed again with normal Ringer's solution, levels of DA declined in all groups, and no treatment-related differences were evident during the remaining 60 minutes of sampling. The increase in DA seen in the first sample (240 minutes time point) after high potassium infusion was due to dead volume of the microdialysis sample collection system.

Analysis of striatal DOPAC levels in the dialysates revealed only a significant effect of sampling time [$F(9, 18) = 13.735$, $p < 0.0001$]. One way ANOVA at each time point did not show any difference among groups in DOPAC concentration during the course of the experiment (Figure 5B). Similarly, analysis of HVA levels showed only a significant effect of sampling time [$F(9, 18) = 6.074$, $p < 0.0001$] but no effect of group or group x sampling time (Figure 5C).

Administration of vehicle (1% methylcellulose) or 100 mg/kg of ATR did not result in acute observable effects in these rats, while some rats injected with 200 mg/kg ATR exhibited hypoactivity during the first two hours after injection, after which levels appeared normal. Histological analysis confirmed that cannula placement for all rats were

appropriately located in dorsal striatum.

DISCUSSION

The current study demonstrates that sustained low level ATR exposure in diet can adversely affect both major long-length DA tracts of the central nervous system, resulting in persistent increases in locomotor activity, alterations in responsivity to the indirect DA agonist amphetamine, changes in monoamine levels, and, ultimately, loss of neurons in the midbrain. Thus, adverse effects of ATR are not restricted to endocrine and reproductive systems, nor to hypothalamic regions of brain. The effects observed here cannot be ascribed to acute toxicity, since the half-life of ATR in tissue ranges from 31.3 to 38.6 hours, and 95 % of the ATR administered is excreted within 7 days of dosing while changes in monoamines and numbers of neurons were measured two weeks post-treatment. Moreover, the doses used here did not produce any changes in body weight or food consumption or any signs of overt toxicity.

The observations of protracted changes in neurotransmitter levels coupled with neuronal loss have particular significance given the critical roles of the nigrostriatal and mesocorticolimbic DA systems in controlling fine motor behavior and complex cognitive function, respectively (Clark et al. 2004; Crossman 2000; Remy and Samson 2003). Dysfunctions of DA systems include Parkinson's disease, schizophrenia, attention deficit disorder and learning and memory impairments. Collectively, the current findings raise the possibility that ATR exposure could be a contributory risk factor for such disorders.

Chronic atrazine exposure caused cell loss not only to TH^+ immunoreactive cells but also to TH^- cells in the VTA and SNpc. The non-dopaminergic neuronal subpopulation in these regions includes GABAergic (Deniau et al. 1978), calbindin (Gerfen et al. 1985), cholecystokinin-like immunoreactive neurons (Seroogy and Fallon

1989). The lack of selectivity of effects makes it likely that ATR will exhibit neurotoxicity, including cytotoxicity to other neuronal populations in other brain regions as well, although other regions were not examined in this particular study. Additionally, ATR may exert neurotoxic effects on other cell types of the brain as well, such as glial cells. The specificity and mechanism(s) of atrazine effects within the CNS remains to be determined and such assessments are clearly warranted based on the findings presented here.

Chronic ATR increased locomotor activity, effects which were present after three months of exposure, persisted for six months, and were still evident even two weeks after cessation of exposure. Moreover, rats treated for two months with 10 mg/kg ATR exhibited an enhanced locomotor activity response to a d-amphetamine challenge. Amphetamine is known to promote the release of DA and a decrease in its re-uptake into the pre-synaptic terminal (Cooper et al. 2003). Thus the increases in locomotor activity could reflect ATR-induced upregulation of striatal DA receptors, as might be expected to occur in response to the corresponding reduction in basal DA levels (Figure 3A) or DA release produced by ATR (Figure 5A). Placement in a novel environment such as the locomotor activity chamber could increase DA, activating upregulated DA receptors and thereby produce hyperactivity (Badiani et al. 1998), a hypothesis in agreement with the increases in locomotor activity induced by amphetamine sulfate (Mao et al. 2001).

The locomotor hyperactivity observed here differs from findings of a previous study in which 1000 mg/kg of ATR administered for 4-11 days decreased rearing in the open field (Ugazio et al. 1991). Such decreases could reflect acute toxicity of a high dose of ATR, since the chemical was administered immediately before the behavioral

evaluation in that study, coupled with a decline in DA release that would accompany its administration and be expected to reduce activity levels, as was observed.

The reductions noted here in levels of DA, NE and 5-HT observed respectively, in striatum, prefrontal cortex and hypothalamus at the 10 mg/kg ATR dose could be due to inhibitory effects on synthesis in these monoamine pathways. Precursors of DA and norepinephrine (tyrosine) and of serotonin (tryptophan) undergo the same hydroxylation process via tyrosine hydroxylase or tryptophan hydroxylase, respectively. Both enzymes are pteridin-dependent aromatic amino acid hydroxylases and are highly homologous, reflecting a common evolutionary origin from a single genetic locus (Cooper et al. 2003). In an *in vitro* study using PC12 cells in which NE and DA were decreased by ATR, the NE effect was reversed when cells were co-incubated with agents known to enhance transcription and phosphorylation of DBH and tyrosine hydroxylase (Das et al. 2003), consistent with the possibility that ATR may have inhibitory effects on these enzymes.

Previous studies have reported changes in hypothalamic DA and/or NE levels following acute ATR administration at 100 mg/kg by gavage to male rats (Cooper et al. 1998). Such changes were not observed in the chronic exposure paradigm used here, a difference that could reflect initiation of compensatory mechanisms to maintain a constant production of these neurotransmitters under conditions of chronic exposure. Alternatively, catecholamine levels were determined in specific hypothalamic nuclei in that study, while here the hypothalamus was examined in its entirety, thus possibly diluting any regional changes (Cooper et al. 1998).

Chronic ATR exposure did decrease hypothalamic 5-HT (serotonin) levels, effects consistent with its known alterations of neuroendocrine systems, including the

release of luteinizing hormone (LH) and prolactin (PRL). Serotonergic neurons from the dorsal and medial raphe nuclei project to hypothalamus, activating the hypothalamo-pituitary-adenocortical (HPA) and the hypothalamo-pituitary-gonadal (HPG) axes in the rat (Fuller 1996; Jorgensen et al. 1998). Agents that disrupt serotonin transmission are known to alter the HPA and HPG axes (Fuller 1996; Fuller and Snoddy 1990). Furthermore, selective degeneration of the midbrain dorsal and ventromedial region of the hypothalamus induced by 5,7 dihydroxytryptamine reduces LH levels (van de Kar et al. 1980). Taken together, it can be inferred that reductions in hypothalamic serotonin resulting from ATR could impact both the HPA and HPG axes, and thereby alter other organ systems of the body with which these systems interact.

DA and NE alterations in prefrontal cortex are also notable given the critical role of this structure in mediating executive function including working memory (Dreher et al. 2002). Dysfunction of this region is also involved in cognitive deficits, altered stress responsivity, hyperactivity disorder and schizophrenia (Mostofsky et al. 2002; Tam and Roth 1997; Viggiano et al. 2003). An increase in cortical DA levels, such as observed at 5 mg/kg, could be due to an increase in DA synthesis, decreased degradation or altered reuptake. It is worth noting that autoreceptors on DA terminals in the prefrontal cortex regulate release but not synthesis of DA (Cooper et al. 2003), which may explain why augmented DA concentrations in this region are not corrected following ATR exposure.

Findings from the microdialysis component of these experiments are consistent with such an assertion and show alterations in the dynamics of DA in striatum following acute ATR treatment. A dose-dependent decrease in striatal DA release as observed here would normally trigger compensatory mechanisms such as decreased re-uptake rate, and

increased production and release of DA. As is evident from Figure 5, none of these compensatory mechanisms appear to be operative, at least within the time-frame encompassed by these experiments.

The observed decline in both basal and stimulated DA release could have several explanations. First, it could reflect a generalized inhibition of DA synthesis, given the absence of group differences at the end of the experiment. DA is distributed mainly in two functional presynaptic compartments, a cytoplasmic pool and a vesicular pool. Potassium-induced release is Ca^{+2}-dependent and occurs from the vesicular pool (Du et al. 1999) which is the newly synthesized pool (Lamensdorf et al. 1996). Another possibility is that ATR decreases the firing rate of striatal and/or substantia nigra neurons, decreasing DA release. Additionally, tyrosine hydroxylase exists in two kinetics forms, with differential affinities for tetrahydrobiopterin (cofactor for TH). The proportion of TH in the high affinity state appears to be a function of neuronal firing rate (Cooper et al. 2003). A dose of 100 mg/kg ATR decreased cerebellar cell firing rate after 60 and 90 minutes, with rates returning to normal by 180 minutes; this inhibitory effect lasted up to 180 minutes following exposure to 200 mg/kg ATR (Podda et al. 1997). ATR could also be acting on ionotropic GABA receptors. Binding of RO15-4513 (an inverse agonist of the GABA A receptor benzodiazepine site) was inhibited when cortical membranes were incubated with ATR (Shafer et al. 1999). This would increase the influx of chloride ions leading to hyperpolarization of cells, preventing depolarization which would, in turn, decrease DA release.

No changes in levels of the DA metabolites DOPAC and HVA were observed in the microdialysis component of this study, although DA levels were altered. DA is

converted to DOPAC intraneuronally after reuptake, while extraneuronal DA is converted to HVA by the enzymes COMT and MAO. The lack of change in DOPAC and HVA could reflect the relatively modest nature of the changes in DA, leaving a sufficiently high concentration of DA in the intracellular space to maintain constant levels of DOPAC and HVA production. Further, DA reuptake was not impaired. The decreases in DOPAC and reductions in extracellular DOPAC and HVA following potassium stimulation across sampling time in the microdialysis component of this study agree with results of other such studies (Holson et al. 1998; Robinson and Camp 1991; Stanford et al. 2000; Westerink and Tuinte 1986) and may reflect initial damage caused by the probe insertion, which recovers after several hours. The decrease in DOPAC levels that occurs with the increase in extracellular DA after potassium perfusion is thought to reflect a decrease in intracellular DA metabolism by monoamine oxidase (Camarero et al. 2002).

At the current time, our understanding of the health-related impacts that ATR exposures may exert in human populations remains unknown. Assessments of occupational exposures have been limited and systematic studies of environmental exposures have not been undertaken. While the doses of ATR used in these studies are higher than those estimated for human exposures, there are additional considerations that must be applied to such comparisons. First, data projecting human exposure is always limited, since it will be dependent upon when exposures occurred relative to the time of measurement, and does not provide measurement of the tissue of interest, i.e., the brain, in such cases. Secondly, the doses used here are low for the experimental species (rat), being consistent with previously reported NOAEL and LOAEL levels. Furthermore,

even at these levels, the doses may not have been as high as administered, since probably not all the ATR ingested would have been absorbed since a portion of it could have been easily eliminated through the feces, suggesting that actually lower absorbed doses could underlie the deleterious effects observed in this study.

In summary, the collective findings from this study demonstrate that ATR may have broad effects on brain monoamine systems and thereby influence a wide range of behavioral functions. Clearly, additional studies will be needed to unravel the targets of ATR and the mechanism(s) of its effects, as well as the ultimate human health consequences of such exposures for behavioral and/or neurological dysfunctions.

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Figure Legends

Figure 1. A) Experimental design for the chronic ATR exposure component of the study, and B) acute ATR exposure design for microdialysis studies. Abbreviations: SNPc = substantia nigra pars compacta; VTA = ventral tegmental area; K+ = potassium; MC = methylcellulose; Ringer's = normal Ringer's solution.

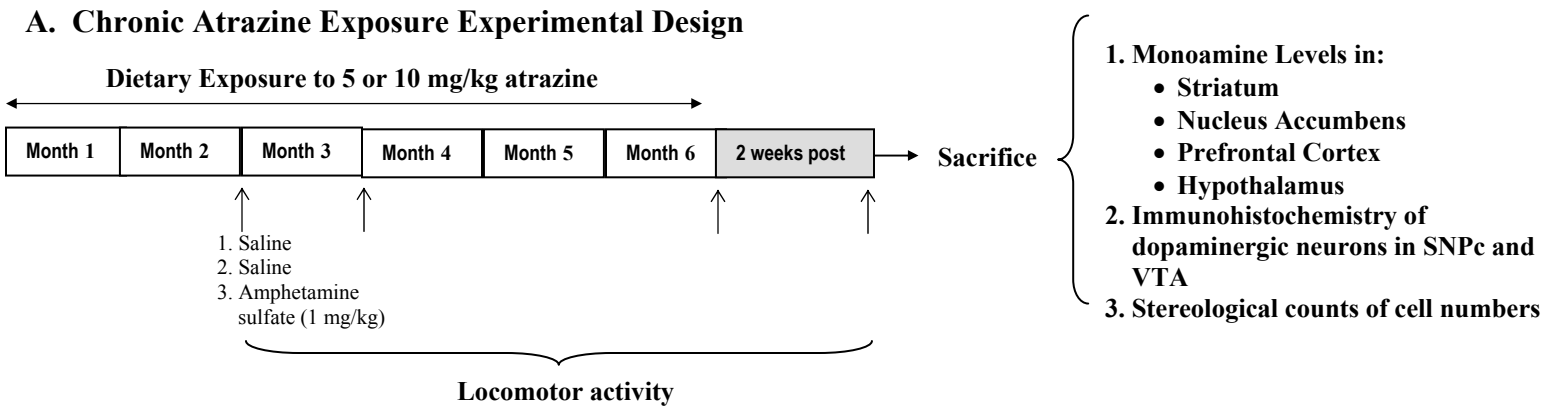
Figure 2. Horizontal locomotor activity (group mean as percent of control ± SEM).
A) Effect of a 1 mg/kg dose of amphetamine sulfate administration on locomotor activity after two months of atrazine exposure. B) Spontaneous locomotor activity measured at three months and six months of atrazine exposure, and two weeks post-termination of atrazine exposure. One-way ANOVAs at each time-point were followed by Fisher post hoc test. (*) Indicate significantly different from control group at $p < 0.05$ (n= 8-10 rats per treatment group). Absolute values (total counts ± SEM) of control animals are: 8094.44 ± 1822.95 (amphetamine sulfate challenge after two months of ATR exposure); 3860.56 ± 703.66 (three months of ATR exposure); 3168.60 ± 550.36 (six months of ATR exposure) and 4257.00 ± 588.45 (two weeks post-termination of atrazine).

Figure 3. Levels of DA (3A), serotonin (3B) and norepinephrine (3C) (group mean as percent of control ± SEM) in striatum, prefrontal cortex, nucleus accumbens and hypothalamus two weeks after cessation of atrazine exposure (6 months). One-way ANOVAs at each structure were followed by Fisher post hoc test. (*) Indicate significantly different from control group at $p < 0.05$ (n= 7-10 rats per treatment group). Absolute values (ng/mg protein ± SEM) for control animals are: DA: 122.25 ± 8.54

(striatum), 2.83 ± 0.21 (prefrontal cortex), 6.09 ± 0.55 (hypothalamus), 78.70 ± 7.12 (nucleus accumbens). 5-HT: 2.46 ± 0.27 (striatum), 15.45 ± 1.31 (prefrontal cortex), 14.44 ± 0.46 (hypothalamus), 8.22 ± 0.68 (nucleus accumbens). NE: 11.44 ± 0.77 (prefrontal cortex), 34.65 ± 1.67 (hypothalamus), 1.63 ± 0.14 (nucleus accumbens).

Figure 4. Numbers of TH^+, TH^- or total cells in substantia nigra pars compacta (SPNc; top row) or in ventral tegmental area (VTA; bottom row) measured using unbiased stereology and determined following a two-week post-termination of atrazine treatment period. Each dot depicts values for an individual animal, with n=5 randomly selected per treatment group counted. Bar represents median of group.

Figure 5. Time course (group mean as percent of basal release ± SEM) of striatal release of DA (A), DOPAC (B), and HVA (C) over the course of microdialysis. Microdialysates were collected every 30 minutes; high potassium infusion (60 mM) lasted 1 hour, after which normal Ringer's solution was restored for one more hour. (*, $^+$) Indicate significantly different from control group at $p < 0.05$ (n= 7-8 rats per treatment group). The presence of response in first sample after high potassium was due to the dead volume of the microdialysis sample collection system.


A. Chronic Atrazine Exposure Experimental Design
Dietary Exposure to 5 or 10 mg/kg atrazine
Month 1
Month 2
Month 3
Month 4
Month 5
Month 6
2 weeks post
Sacrifice
1. Saline
2. Saline
3. Amphetamine sulfate (1 mg/kg)
Locomotor activity
1. Monoamine Levels in:
• Striatum
• Nucleus Accumbens
• Prefrontal Cortex
• Hypothalamus
2. Immunohistochemistry of dopaminergic neurons in SNPc and VTA
3. Stereological counts of cell numbers

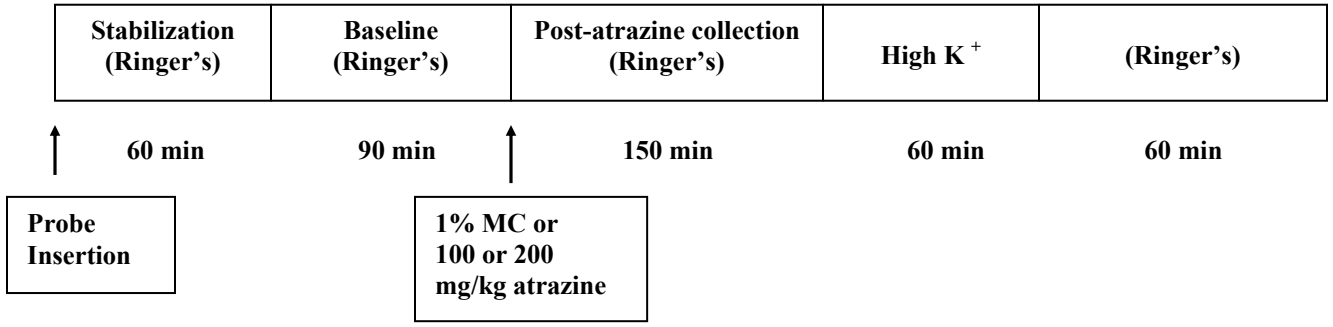

B. Acute Atrazine Exposure and Microdialysis
Stabilization (Ringer's)
Baseline (Ringer's)
Post-atrazine collection (Ringer's)
High K +
(Ringer's)
60 min
90 min
150 min
60 min
60 min
Probe Insertion
1% MC or 100 or 200 mg/kg atrazine

Figure 1

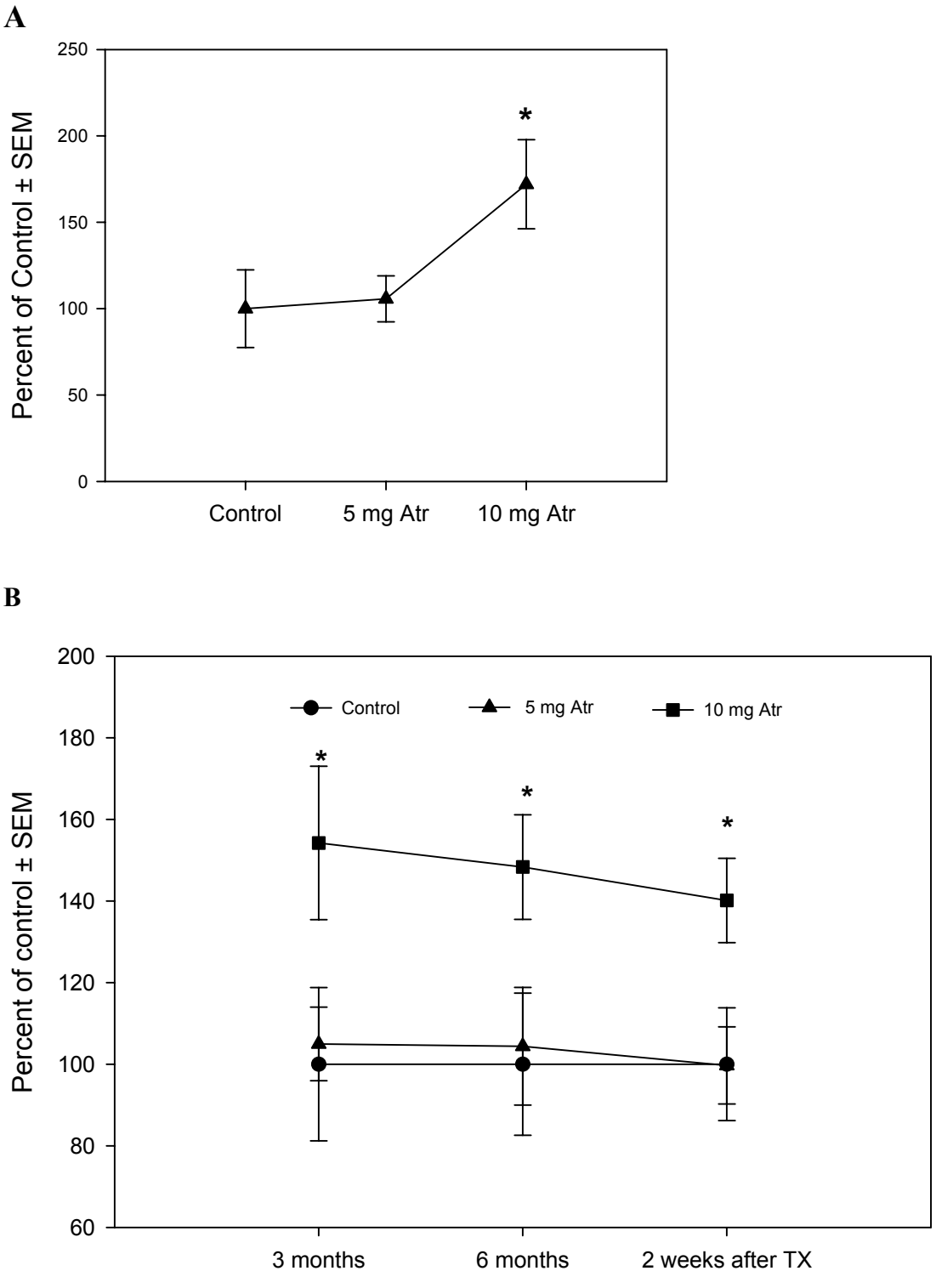

A
250
200
150
100
50
0
Percent of Control ± SEM
*
Control
5 mg Atr
10 mg Atr
B
200
180
160
140
120
100
80
60
Percent of control ± SEM
Control
5 mg Atr
10 mg Atr
*
*
*
3 months
6 months
2 weeks after TX

Figure 2

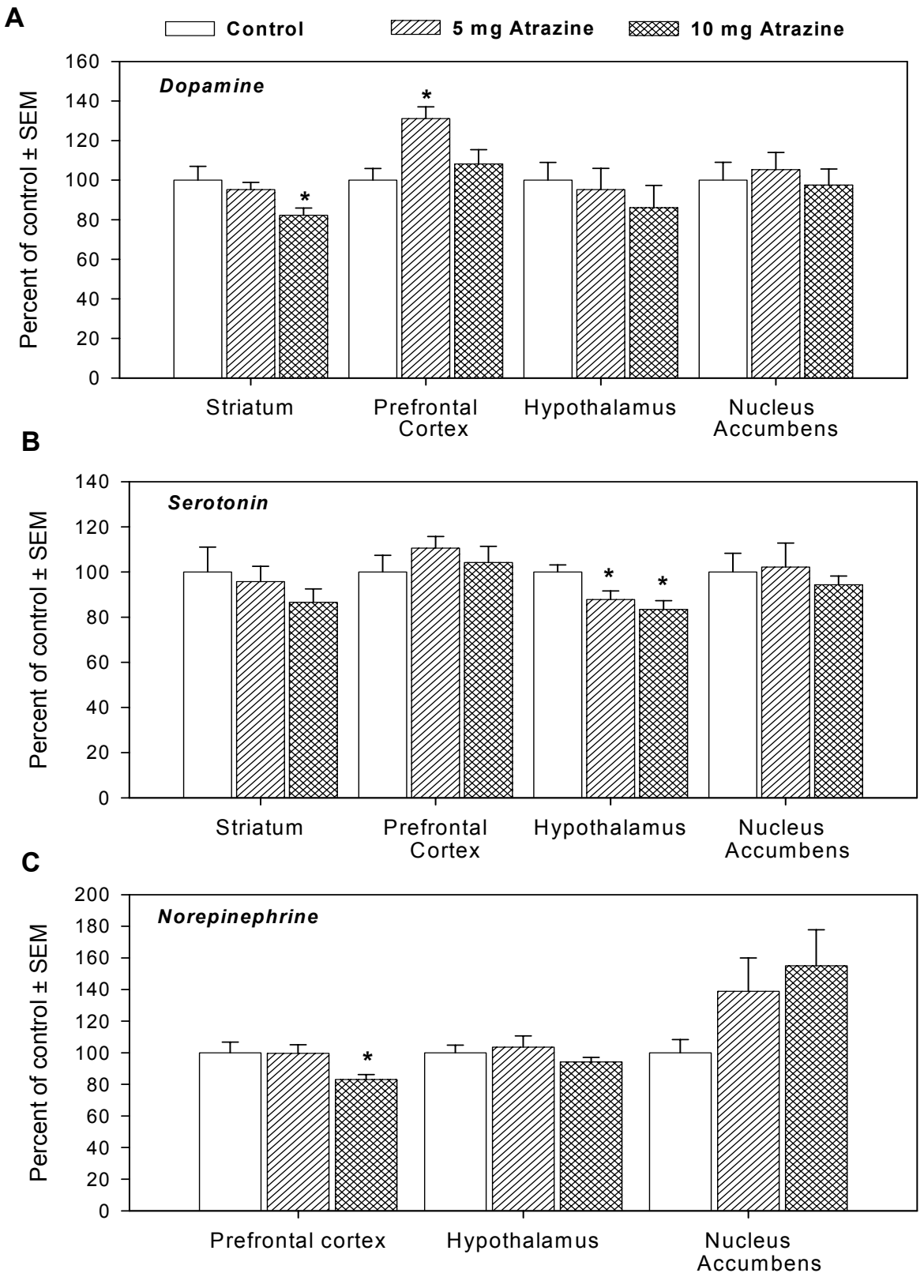

A
Control
5 mg Atrazine
10 mg Atrazine
Dopamine
Percent of control ± SEM
160
140
120
100
80
60
40
20
0
*
*
Striatum
Prefrontal Cortex
Hypothalamus
Nucleus Accumbens
B
Serotonin
Percent of control ± SEM
140
120
100
80
60
40
20
0
*
*
Striatum
Prefrontal Cortex
Hypothalamus
Nucleus Accumbens
C
Norepinephrine
Percent of control ± SEM
200
180
160
140
120
100
80
60
40
20
0
*
Prefrontal cortex
Hypothalamus
Nucleus Accumbens

Figure 3

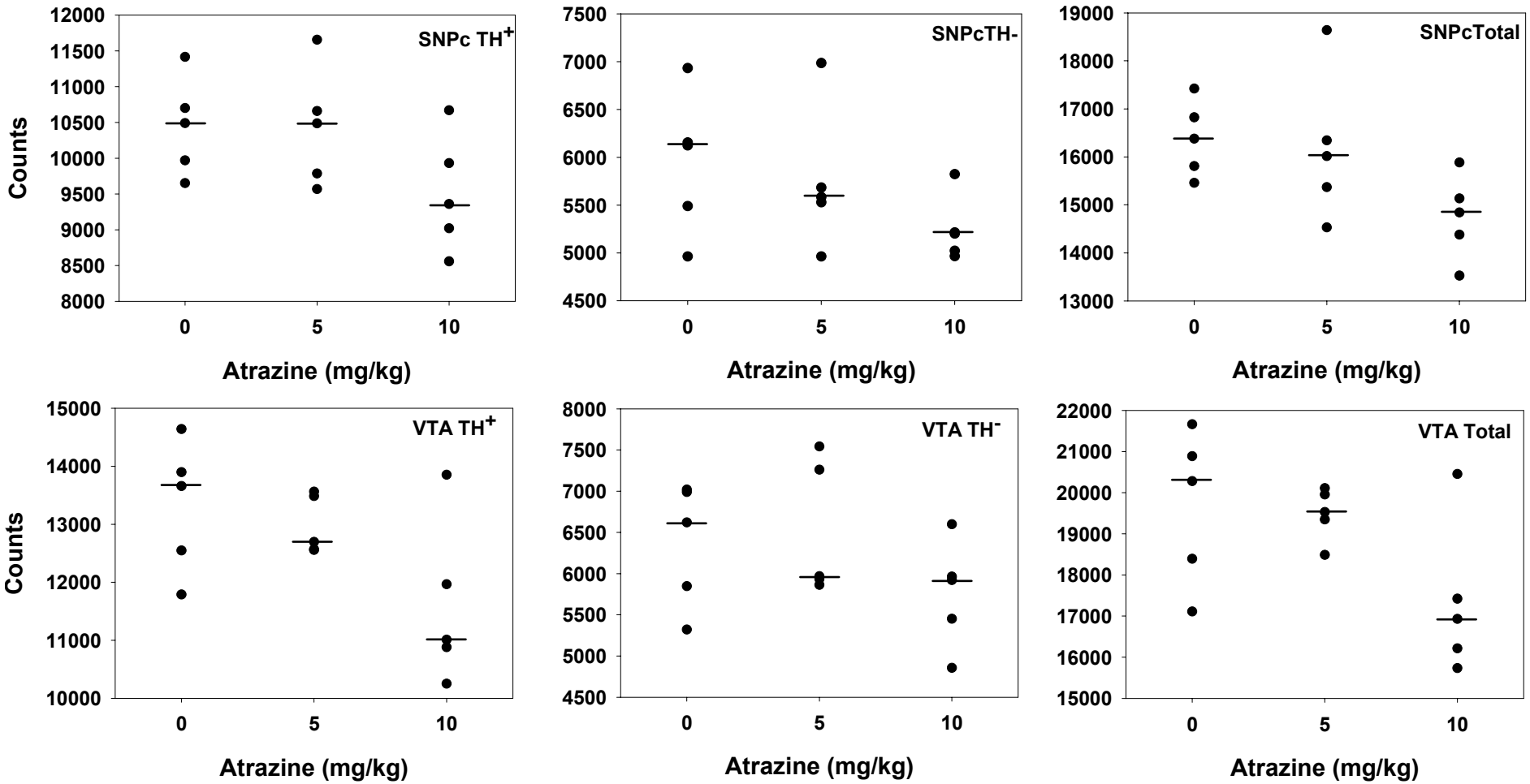

SNPc TH+
SNPcTH-
SNPcTotal
VTA TH+
VTA TH-
VTA Total
Counts
Atrazine (mg/kg)
0
5
10

Figure 4

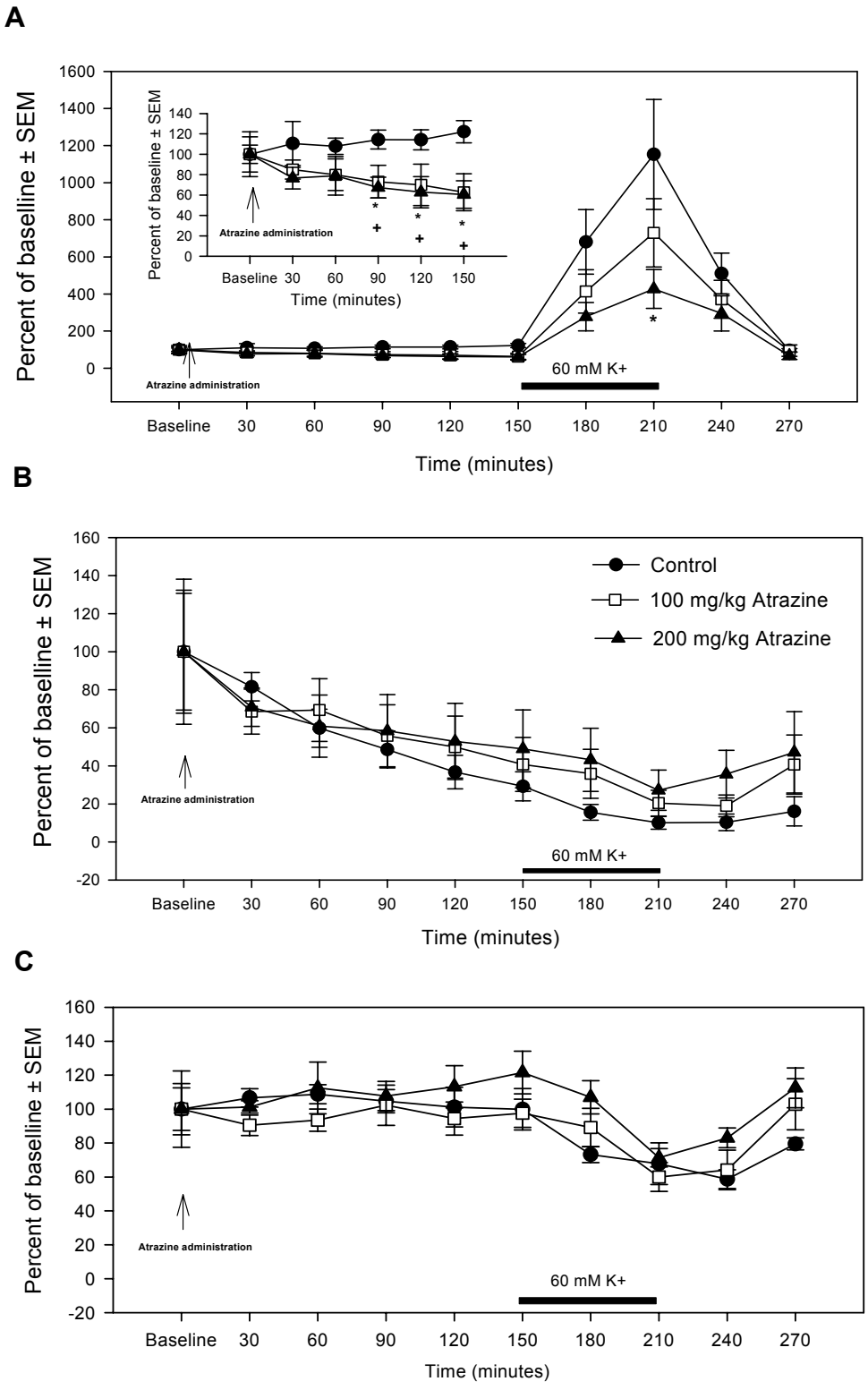

A
Percent of baselline ± SEM
Percent of baseline ± SEM
Time (minutes)
Atrazine administration
60 mM K+
B
Control
100 mg/kg Atrazine
200 mg/kg Atrazine
Percent of baselline ± SEM
Atrazine administration
60 mM K+
Time (minutes)
C
Percent of baselline ± SEM
Atrazine administration
60 mM K+
Time (minutes)

Figure 5